PROGRAM AND ABSTRACTS OF PAPERS

Western Experiment Station Collaborators Conference

on

THE IMPORTANCE OF MOLD METABOLITES IN AGRICULTURAL PRODUCTS

March 1 to 3, 1965

Western Regional Research Laboratory
800 Buchanan Street
Albany, California

Western Utilization Research and Development Division
Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

415391

PROGRAM
WESTERN EXPERIMENT STATION COLLABORATORS' CONFERENCE
on
The Importance of Mold Metabolites in Agricultural Products
March 1-3, 1965

Monday, March 1

9:30 A.M. MORNING SESSION

Abstract on page

Chairman: Floyd DeEds, Chief, Pharmacology Laboratory, WURDD, ARS, USDA
Introduction and Welcome: M. J. Copley, Director, WURDD, ARS, USDA
Remarks: F. R. Senti, Deputy Administrator, ARS, USDA

1:30 P.M. AFTERNOON SESSION

Chairman: George M. Briggs, Chairman, Department of Nutritional Sciences, University of California, Berkeley

Chairman: J. Forgacs, Staff Microbiologist, Good Samaritan Hospital, Suffern, New York

Tuesday, March 2

9:30 A.M. MORNING SESSION

Chairman: A. C. Keyl, Head, Pharmacodynamics Investigations, Pharmacology Laboratory, WU

1:30 P.M. AFTERNOON SESSION

Chairman: L. A. Goldblatt, Chief, Oilseed Crops Laboratory, SU, New Orleans, La.

Chairman: C. W. Hesseltine, Head, ARS Culture Collection Investigations, Fermentation Laboratory, NU, Peoria, Ill.

Wednesday, March 3

Tour of Laboratory -- Individual Conferences

FACTORS AFFECTING THE PRODUCTION OF FUNGI OF MATERIALS TOXIC TO ANIMALS

C. M. Christensen
University of Minnesota, St. Paul

Grains, grain products, and other materials consumed by man and other animals are subject to invasion by a great variety of fungi. Whether a given fungus will or will not produce metabolites toxic to those consuming the material on which the fungus has grown depends on a number of factors. Among them are (1) the strain of the fungus, (2) the substrate on which it grows, (3) the temperature at which it grows, (4) the length of time it grows, (5) whether it is growing alone or in combination with other fungi, and (6) the kind of animal to which it is fed. Most of these factors are interacting and tend to make the elucidation of some toxicoses tremendously complex.

TOXIC FUNGI OF *CYCAS CIRCINALIS*

J. Forgacs
Good Samaritan Hospital, Suffern, New York

INVESTIGATION OF AFLATOXIN FORMATION BY *ASPERGILLUS FLAVUS*

C. W. Hesseltine
Fermentation Laboratory, Northern Utilization Research
and Development Division, Peoria, Illinois

Aflatoxin can be produced by *Aspergillus flavus* either on solid or liquid media (still or shaken) made from potato dextrose. Aflatoxin occurs in culture filtrates, mycelium, and spores. Examination indicates that certain strains, e.g. NRRL 482, do not produce aflatoxin under the conditions used, whereas others vary greatly in the amounts formed. Aflatoxin is produced as early as 48 hrs., but maximum yields are usually obtained in fermentation liquors in 5 days. Often amounts in culture liquors decrease rapidly after 5 days. Associated with toxin formation is a drop in pH to 3 when some of the best producers, such as NRRL 2999 and NRRL 3000, are used. Examination of 52 strains of *A. oryzae* obtained from pure culture starters, used in Japan to manufacture shoyu, miso, and black beans, showed 37 that produced fluorescent material, but there was no evidence that the fluorescing substances were aflatoxin. A number of good food samples including miso, shoyu, and various types of tempeh were examined, and although they had a great deal of fluorescent material, none contained aflatoxin at levels detectable by the methods used.

ANALYTICAL METHODS FOR AFLATOXIN

L. A. Goldblatt
Oilseed Crops Laboratory, Southern Utilization
Research and Development Division, New Orleans, Louisiana

Two main groups of test methods have been developed for determination of aflatoxin, one biological, the other chemical. The biological tests include use of ducklings, chick embryos, microorganisms, enzymes, and cell mitosis. The chemical tests are based on specific physical or chemical properties, especially fluorescence, of purified aflatoxin.

The most widely used biological test, that with day-old ducklings, utilizes a relatively crude extract, affords a specific toxicological response, and can be made roughly quantitative but does not distinguish between the four related toxic components of the mixture commonly referred to as aflatoxin. The most widely used chemical tests are based on quantitative extraction of aflatoxin by means of a solvent, partial purification to remove interfering components, separation of individual aflatoxin components by chromatography, and finally quantification, generally by comparison of intensity of fluorescence when illuminated with ultraviolet radiation.

Chemical tests will be emphasized. Various solvent systems employed for extraction, techniques used for removal of interfering substances, procedures for chromatographing and for final estimation of the content of individual aflatoxin components will be described and some of their relative merits evaluated. Attention will also be given to the precision and limits of detection of aflatoxin afforded by the various procedures. Modifications permitting micro or semi-micro scale operation, e.g. for determination of the aflatoxin content of a part of a single peanut kernel, will be described.

MEASUREMENT OF AFLATOXIN IN CEREAL GRAINS

Odette L. Shotwell
Fermentation Laboratory, Northern Utilization Research
and Development Division, Peoria, Illinois

A survey for the presence of aflatoxin in the following agricultural commodities has been initiated: wheat, corn, sorghum, oats, and soybeans. Additionally, all the grains except oats were tested under ideal conditions as substrates for aflatoxin production with three strains of Aspergillus flavus and compared to peanuts and rice, the more commonly known substrates. Aflatoxins were produced on sorghum at levels comparable to those obtained on peanuts. The least satisfactory substrate was soybeans even though the A. flavus strains used grew well. When the solid substrates, wheat and corn, were properly aerated, high yields of aflatoxin were obtained.

Samples for the survey are being collected from carloads of grain at different markets by the Grain Division of USDA's Agricultural Marketing Service. Statistically, 300 samples of each grain must be collected at each selected location in order to get a significant yes or no answer. A total of 3,900 samples will be collected of the various grains. The method of analysis used is the FDA "short procedure." As of February 1, 192 samples have been analyzed. Among these only extremely low levels of aflatoxin were detected in several samples of corn, sorghum, and oats.

AFLATOXICOSIS IN RAINBOW TROUT

John E. Halver
Western Fish Nutrition Laboratory
U. S. Department of Interior, Cook, Washington

Three large-scale studies were organized to study primary or secondary vectors in rainbow trout hepatomagenesis. Thirteen classes of mammalian carcinogens were individually incorporated into a test diet of casein 35, gelatin 15, white dextrin 29, carboxymethyl-cellulose 4, alpha cellulose 4, minerals 4, cod liver oil 2, corn oil 6, vitamins 1, and were fed to paired lots of initial feeding rainbow trout at 1/16, 1/4, 1, 4, and 16X normal rodent dosage. Toxic effects as expected terminated high-level study of 2-acetylaminofluorene, aminoazotoluene, carbon tetrachloride, DDT, p-dimethylaminoazobenzene, diethylstilbesterol, tannic acid, thioacetamide and thiourea. Histopathologically confirmed hepatoma appeared in fish fed intermediate or low levels of 2-acetylaminofluorene, aminoazotoluene, carbarsone, carbon tetrachloride, DDT, diethylstilbesterol, dimethylnitrosamine, p-dimethylaminoazobenzene, tannic acid, thiourea and urethane. In concurrent studies, fish fed fat extracted by Bloor-LaRoche technique from suspect commercial ration showed loci of acidophilic or fatty degeneration of adenocarcinoma or trabecular hepatocellular hepatoma.

Small trout force fed high levels (0.03 to 0.3 mg./kg. per day) of thin-layer-chromatogram-purified aflatoxin B_1 for 5 days died with acute aflatoxicosis at 8 to 15 days. Autopsy showed histologically confirmed widespread congestion with extravasated blood in livers, visceral fat and digestive tracts. Long-term chronic feeding studies in 15°C. water with crude or purified aflatoxins added to test diet above showed fish with classical hepatoma after 6 to 9 months. Trout fed crude material at 40 ppb of diet had most livers with classical multinodular hepatocellular carcinoma. Lower levels of diet insult showed lower incidence of hepatoma. Histologically, Bouin's fixed, H & E stained, skip-serial sectioned tissues showed discrete, rounded, basophilic, trabecular hepatoma similar to those seen in fish fed chemical carcinogens.

Concurrent bioassay of fat Bloor:LaRoche-extracted from five different area cottonseed meals processed by three standard methods (SP, PPS, S) showed hepatoma in those cottonseed meals carrying high aflatoxin load levels by chemical assay. Negative control diet fed 3 strains of fish continued historically negative throughout 4 different hepatoma induction series of experiments over a five-year period.

OBSERVATIONS ON HEPATOMA IN RAINBOW TROUT

R. O. Sinnhuber
Department of Food Science and Technology
Oregon State University, Corvallis

In 1963, Wolf and Jackson of California implicated cottonseed meal in a commercial dry trout ration as the agent responsible for hepatoma in rainbow trout. The identity of the trout liver carcinogen and whether it was intrinsic or extrinsic to cottonseed meal were unknown. Rainbow trout feeding trials, currently underway, confirm the findings of Wolf and Jackson, and a similar commercial formula produced a 100% incidence of hepatocarcinoma in 12 months.

Chemical assay techniques were developed which employ extraction by selective solvents, thin layer chromatography, and fluorescence densitometry. These determinations revealed that the cottonseed meal present in the commercial ration contained aflatoxins. This identical cottonseed meal and lipid extracts thereof, as well as aflatoxin, when fed in a purified test diet to rainbow trout, produced a significant incidence of liver cancer. Confirmatory tests using ducklings were positive.

Cottonseed flour and fish and cottonseed meals which were free of aflatoxins did not produce hepatoma. Gossypol and cyclopropenoid fatty acids, intrinsic to cottonseed, were not found to be hepatomagenic. The commercial trout ration, without cottonseed meal, also produced liver cancer. The dietary component which is involved and the identity of the carcinogen is the subject of our present investigations. It is apparent from these studies that rainbow trout is an extremely sensitive test animal for liver carcinogens, especially aflatoxins. This investigation is supported by Public Health Service Research Grant No. CA-06825 from the National Cancer Institute.

BIOCHEMICAL EVALUATION OF AFLATOXIN

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Research and Development Division, Albany, California

Several procedures are available for the detection and estimate of aflatoxin in agricultural products. In general these are based on concentration of this toxin from suitable extracts by partitioning between appropriate solvent phases and examining the partially purified extracts by thin layer chromatography. Once aflatoxin is detected in extracts, its identity can be confirmed by chromatography after it is converted to certain addition derivatives. These chemical tests, however, need to be verified by a bioassay such as the crop-tubing of extracts to ducklings or injection into the chick embryo. A satisfactory technique employing open-shell week-old duck embryos has been used in this laboratory.

Another bioassay has been developed here for the direct testing of meals without need of extracts. The total meal is fed for a period of 1 to 2 weeks to ducklings at a high level in the ration. Mortality, growth retardation and liver histopathology have been found to be correlated with the chemical estimate of aflatoxin in the meals. Further confidence in this method was derived from three types of experiments: (a) Growth retardation and liver histopathology were observed when TLC-purified aflatoxin B_1 was added at different known levels to a synthetic diet. (b) A toxic meal (about 1 ppm B_1) which killed nearly all the ducklings by the end of the 2-week period of the test and caused severe growth retardation, bile duct proliferation and parenchymal cell damage, was tested with this method after it had been extracted with an efficient solvent for aflatoxin. Almost complete removal of the toxicity was indicated by the biological test: no deaths occurred and growth was satisfactory. (c) This toxic meal was tested after treatment of the meal with ammonia. It is known that aflatoxin is unstable in alkali or acid media. The results of the biological test indicate elimination of the toxicity as a result of this treatment.

In other experiments, the relationship of structural modification of crystalline aflatoxin B_1 to its physiologic action was investigated by the crop-tubing method in an attempt to study the mechanism of its action and means of modifying this action. It is known that aflatoxin forms addition products with hydroxylated solvents such as formic or acetic acids in the presence of a strong acid catalyst. The addition occurs across the double bond of the enol ether of the furano ring moiety of aflatoxin. The biological tests indicate that the conversion of aflatoxin to these addition products appears to be accompanied by loss of activity, pointing to the importance of the intact enol ether system for the action of aflatoxin.

Finally, a preliminary experiment was carried out in the rat to study tissue distribution and metabolism of aflatoxin B_1-C^{14} obtained from mold given formate-C^{14}. Chromatographic-radioautographic evidence shows appreciable excretion in the urine and feces. Small amounts of aflatoxin, however, were detectable in chromatograms of tissue extracts two days after the rat received the dose.

PUBLIC HEALTH ACTIVITIES FROM AFLATOXINS

J. W. Bell
Bureau of Food and Drug Inspections
California State Department of Public Health, Berkeley

APPROACHES TO REMOVE OR INACTIVATE AFLATOXIN ON COTTONSEED AND PEANUTS

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The first and perhaps best approach to minimize contamination with aflatoxin in cottonseed and peanuts is prevention. Suitable harvesting and handling procedures to avoid mechanical damage should be practiced. Adequate storage facilities to prevent opportunity for mold growth should be available. Mechanical methods for separation of contaminated seed may be effective. Methods which merit consideration include, in the case of peanuts, culling on the basis of physical characteristics such as size, obvious damage and discoloration. Additional segregation after splitting may be necessary. In the case of cottonseed, segregation on the basis of density may be of value.

Removal of aflatoxin by means of solvents when processing cottonseed and peanuts to oil and meal presents good potential. Selective solvents which remove both oil and aflatoxin from residual meal may be used since the aflatoxin can be subsequently removed from the oil by conventional refining and bleaching. Selective solvents which remove only the aflatoxin may also be used on prepared cottonseed and peanut meats or on the extracted meal. The potential utility of chemical treatments during processing of cottonseed and peanuts to oil and meal as well as treatments applied to extracted meats, with or without added heat, will be discussed.

TOXIC METABOLITES OF FUSARIA

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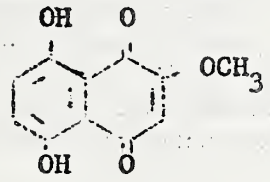
The chemistry and physiological properties of several naphthalenic compounds isolated from wilt-producing Fusarium sp. will be discussed. The structures of javanicin, fusarubin and rubrofusarin have been elucidated by work of several groups. Isolation and elucidation of structures of two new naphthaquinones from culture filtrates of a Fusarium sp. will be reported. All five of these compounds are closely related chemically and, presumably, biogenetically: all but rubrofusarin contain the 2-methoxynaphthazarin system (I). Metal chelating properties (Al, Mg, Ni, Cu) of the naphthazarins, which were utilized in isolation of the new metabolites, may be of physiological significance.



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TOXIC MOLDS OF FESCUE

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A number of toxigenic molds have been isolated from toxic tall fescue obtained from Missouri by the Industrial Crops Laboratory of the Northern Regional Research Laboratory. The question of whether these toxins are etiological agents in "fescue foot," a disease affecting cattle grazing toxic pastures, is currently the subject of a collaborative study by the Northern and Western Regional Laboratories.

By use of suitable extracts of either the toxic fescue hays or cultures of molds isolated from these hays, it has been possible to demonstrate various toxic effects in the rabbit, mouse, and sheep. In the rabbit, topical application of the extracts on the unabraded skin produces hyperemia, edema, hemorrhage, and necrosis. In the mouse, intraperitoneal injections of the particulate-free filtrates of submerged cultures of the toxic molds produce death as a result of massive hemorrhage, both pulmonary and visceral. The most dramatic lesion is central necrosis of lymphocytes in the follicles of the spleen and generalized enlargement of the spleen. Although these two species are useful in screening of hays and mold cultures for toxic metabolites, it has been difficult to relate these pathological findings to the pathology of cattle suffering fescue foot disease.

Force feeding of the total mold cultures by means of a ruminal fistula in the sheep produced results comparable to some of those seen in fescue-intoxicated cattle. The predominant finding was total ruminal paralysis with subsequent anorexia and refusal to drink water. The resulting emaciation and dehydration explains most of the ensuing pathology. This is also the predominant symptom in fescue foot, in which the dramatic gangrene of the tail and extremities is found chiefly at low ambient temperatures.

The next experiments in the series will involve cattle when the production of sufficient quantities of mold toxins is achieved.

IMMUNOLOGICAL METHODS IN THE STUDY OF MYCOTOXINS

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Immunological techniques have been useful in detecting traces of antigenic or haptenic materials undetectable by ordinary microchemical methods. Relationships between species, genera, and orders can often be delineated by such techniques. In 1929 Matsumoto demonstrated immunological relationships between various species of Aspergilli. In 1939 Henrici demonstrated that immunologically distinct endotoxins can be extracted from the mycelia of Aspergillus flavus and Aspergillus fumigatus. Tilden et al. (1960-1963) largely confirmed Henrici's findings and extended them with mouse-toxicity determinations. Cross-protection experiments indicated considerable immunological specificity of the endotoxins of A. flavus and A. fumigatus. Chemical inactivation and toxicological tests confirmed the fact that the flavus and fumigatus endotoxins differed.

Very sensitive immunological tests that are highly discriminatory with respect to specificity are available for the study of proteinaceous endotoxins. These include precipitin ring tests, Schultz-Dale tests, gross anaphylaxis in guinea pigs, passive cutaneous anaphylaxis (PCA) in guinea pigs, complement fixation, etc. All of these tests depend upon precipitating antibodies in immune sera, usually from rabbits.

Nonprotein (nonantigenic) mycotoxins and other antibiotics of low molecular weight, such as aflatoxin and penicillin, are much more difficult to detect by immunological techniques based upon animal sera. However, if a low-molecular-weight substance can be attached to a protein it may, and often does, function as the haptenic group of a complete antigen capable of eliciting antibody formation against the hapten molecule. Such is the case for penicillin V (mol. wt. 350.38). Because of this, penicillin is one of the most potent and lethal of allergens affecting human beings. We have developed a serological method for detecting penicillin allergy (and hence of penicillin) by passive transfer of human penicillin-allergic sera into monkeys.

Antihaptenic sera can be produced in lower animals by injecting "artificial antigens" prepared by coupling small molecules (haptens) to various proteins. Such an approach will be attempted in our attack upon the nonproteinaceous mycotoxins such as the aflatoxins.

Sera from several hundred mold-allergic French patients are also being examined as detection "reagents" at this time.